



MetR-Regulated *Vibrio cholerae* Metabolism Is Required for Virulence

Citation

Bogard, Ryan W., Bryan W. Davies, and John J. Mekalanos. 2012. MetR-regulated *Vibrio cholerae* metabolism is required for virulence. *mBio* 3(5): e00236-12.

Published Version

doi:10.1128/mBio.00236-12

Permanent link

<http://nrs.harvard.edu/urn-3:HUL.InstRepos:10448704>

Terms of Use

This article was downloaded from Harvard University's DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at <http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA>

Share Your Story

The Harvard community has made this article openly available.
Please share how this access benefits you. [Submit a story](#).

[Accessibility](#)

MetR-Regulated *Vibrio cholerae* Metabolism Is Required for Virulence

Ryan W. Bogard, Bryan W. Davies, and John J. Mekalanos

Department of Microbiology and Immunobiology, Harvard Medical School, Boston, Massachusetts, USA

ABSTRACT LysR-type transcriptional regulators (LTTRs) are the largest, most diverse family of prokaryotic transcription factors, with regulatory roles spanning metabolism, cell growth and division, and pathogenesis. Using a sequence-defined transposon mutant library, we screened a panel of *V. cholerae* El Tor mutants to identify LTTRs required for host intestinal colonization. Surprisingly, out of 38 LTTRs, only one severely affected intestinal colonization in the suckling mouse model of cholera: the methionine metabolism regulator, MetR. Genetic analysis of genes influenced by MetR revealed that *glyA1* and *metJ* were also required for intestinal colonization. Chromatin immunoprecipitation of MetR and quantitative reverse transcription-PCR (qRT-PCR) confirmed interaction with and regulation of *glyA1*, indicating that misregulation of *glyA1* is likely responsible for the colonization defect observed in the *metR* mutant. The *glyA1* mutant was auxotrophic for glycine but exhibited wild-type trimethoprim sensitivity, making folate deficiency an unlikely cause of its colonization defect. MetJ regulatory mutants are not auxotrophic but are likely altered in the regulation of amino acid-biosynthetic pathways, including those for methionine, glycine, and serine, and this misregulation likely explains its colonization defect. However, mutants defective in methionine, serine, and cysteine biosynthesis exhibited wild-type virulence, suggesting that these amino acids can be scavenged *in vivo*. Taken together, our results suggest that glycine biosynthesis may be required to alleviate an *in vivo* nutritional restriction in the mouse intestine; however, additional roles for glycine may exist. Irrespective of the precise nature of this requirement, this study illustrates the importance of pathogen metabolism, and the regulation thereof, as a virulence factor.

IMPORTANCE *Vibrio cholerae* continues to be a severe cause of morbidity and mortality in developing countries. Identification of *V. cholerae* factors critical to disease progression offers the potential to develop or improve upon therapeutics and prevention strategies. To increase the efficiency of virulence factor discovery, we employed a regulator-centric approach to multiplex our *in vivo* screening capabilities and allow whole regulons in *V. cholerae* to be interrogated for pathogenic potential. We identified MetR as a new virulence regulator and serine hydroxymethyltransferase GlyA1 as a new MetR-regulated virulence factor, both required by *V. cholerae* to colonize the infant mouse intestine. Bacterial metabolism is a prerequisite to virulence, and current knowledge of *in vivo* metabolism of pathogens is limited. Here, we expand the known role of amino acid metabolism and regulation in virulence and offer new insights into the *in vivo* metabolic requirements of *V. cholerae* within the mouse intestine.

Received 16 August 2012 Accepted 22 August 2012 Published 25 September 2012

Citation Bogard RW, Davies BW, and Mekalanos JJ. 2012. MetR-regulated *Vibrio cholerae* metabolism is required for virulence. *mBio* 3(5):e00236-12. doi:10.1128/mBio.00236-12.

Editor R. John Collier, Harvard Medical School

Copyright © 2012 Bogard et al. This is an open-access article distributed under the terms of the Creative Commons Attribution-Noncommercial-Share Alike 3.0 Unported License, which permits unrestricted noncommercial use, distribution, and reproduction in any medium, provided the original author and source are credited.

Address correspondence to John J. Mekalanos, jmekalanos@hms.harvard.edu.

The Gram-negative bacterium *Vibrio cholerae* is the etiologic agent of cholera, an acute diarrheal disease that affects an estimated 3 to 5 million people annually (1–3). Two *V. cholerae* biotypes have been responsible for all cholera pandemics: the classical biotype, which is thought to have caused the first six pandemics (4), and the El Tor biotype, which is responsible for the current (seventh) pandemic (1). The pathogenic potential of these biotypes depends primarily on two virulence factors: the toxin-coregulated pilus (TCP) and the cholera enterotoxin (CT). TCP is encoded in the genomic TCP pathogenicity island and is required for intestinal epithelium adherence (5), while CT is encoded in the CTX prophage (1, 6, 7) and is responsible for profuse secretory diarrhea (8). *V. cholerae* pathogenesis is a highly orchestrated response to the host environment. A multitude of virulence factors that influence diverse processes, including chemotaxis, biofilm formation, and DNA repair, have been identified as being critical to host infection and dissemination (9–12). Extending the known

repertoire of virulence factors will provide a more complete picture of the *V. cholerae* infectious program.

To identify additional virulence factors in *V. cholerae*, we utilized a top-down approach by conducting an *in vivo* intestinal colonization screen of a panel of mutants defective in genes encoding transcriptional regulators possibly involved in *V. cholerae* virulence (12). The transition from environment to host to infection by a pathogen requires intricate networks of receptors and regulators that rapidly sense the environment and respond by activation of virulence factors (13). Previous studies have shown that disruption of the TCP island regulator ToxT (14) or the membrane-bound master virulence regulator ToxR (15) results in attenuation of virulence in *V. cholerae*. In each case, misregulation of virulence factors due to the disruption of the respective regulators resulted in a colonization defect. Identification of new virulence regulators serves as a starting point to focus our search for new virulence factors. In this report, we identify the methionine transcriptional regulator MetR as a virulence regulator and show

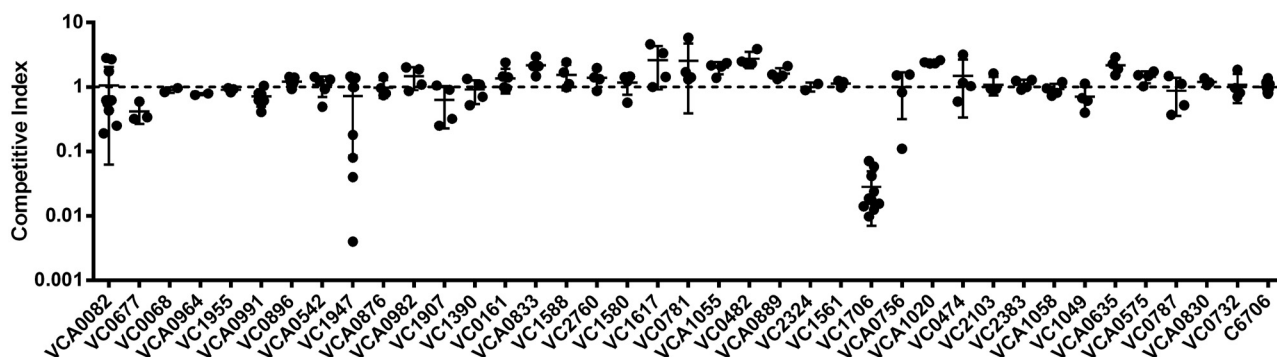


FIG 1 Ability of mutants from the *V. cholerae* LysR-type transcriptional regulator panel to colonize the suckling mouse intestine in competition with wild-type strain C6706. The dotted line indicates a competitive index of 1, and standard deviations (error bars) are presented.

how members of its regulon are critical for *V. cholerae* host colonization. These findings underscore the importance of pathogen metabolism, and the regulation thereof, as a virulence factor.

RESULTS

MetR is essential for colonization of the infant mouse intestine.

We chose to screen the LysR-type transcriptional regulator (LTTR) family because it is the largest and most diverse family of prokaryotic transcriptional regulators with regulatory roles spanning metabolism, cell growth and division, and pathogenesis (16–19). Several LTTRs are conserved among bacteria; thus, virulence regulators identified in *V. cholerae* may also contribute to the virulence of other pathogens.

A panel of 38 *Vibrio cholerae* El Tor mutants representing all annotated LTTRs available from our sequence-defined transposon insertion library was selected (20). The ability of each LTTR mutant to colonize the intestine was examined using the suckling mouse model (21, 22), which compares the *in vivo* fitness of a mutant strain to the wild-type parental strain, yielding a ratio termed the competitive index (CI). Only two LTTR mutants were significantly impaired in colonization (Fig. 1): the strain with the mutation for the methionine regulator MetR showed a highly significant, severe defect ($CI = 0.028 \pm 0.021$; $P < 0.001$), while the strain with the mutation for a regulator of unknown function encoded by the locus VC1947 exhibited a less significant defect ($CI = 0.719 \pm 0.632$; $P < 0.05$). The CI values for well-studied regulators such as GcvA (glycine cleavage regulon), CysB (cysteine regulon), IlvY (branched amino acid regulon), LeuO (leucine regulon), and OxyR (oxidative stress regulon) were not significantly different from that of the wild type (Fig. 1).

glyA1 and metJ are essential for colonization of the infant mouse intestine. Since MetR is a transcriptional activator required for the biosynthesis of methionine, we focused our secondary search for the MetR-regulated virulence factor(s) on a panel of transposon mutants with disruption of genes involved in methionine metabolism. The metabolic pathways in *V. cholerae* remain understudied; however, the well-conserved nature of metabolic genes combined with the well-defined pathways in *Escherichia coli* allowed bioinformatic extrapolation and annotation of the *V. cholerae* genome (23, 24). The KEGG and VchoCyc databases proved useful in determining which pathways may exist in *V. cholerae* and subsequently which mutants to select for the panel (25–27). Colonization assays on our selected mutant panel (Fig. 2) revealed that mutants defective in *glyA1*, which encodes serine

hydroxymethyltransferase, and *metJ*, which encodes the methionine repressor, displayed significant colonization defects with CI values of 0.087 ± 0.075 ($P < 0.001$) and 0.025 ± 0.016 ($P < 0.001$), respectively. Disruption of *metA*, *metB*, and *metC* of the homocysteine biosynthetic branch, *metF* of the folate branch, and the methionine synthase genes *metE* and *metH* did not result in a detectable colonization defect. Additionally, disruption of genes involved in the uptake of methionine, *metI* and *metT*, or cobalamin, *mtsB* and *btuB*, had no impact on *in vivo* fitness. Interestingly, *V. cholerae* possesses a heterologous copy of serine hydroxymethyltransferase encoded by *glyA2* in the small chromosome; however, disruption of this gene did not result in a colonization defect. We hypothesized that the colonization defect displayed by the *glyA1* mutant was the result of glycine limitation; however, the possibility that *glyA1* disruption impedes biosynthesis of a vital glycine-dependent metabolite required further investigation. We decided to examine genes of metabolic pathways involved in glycine processing: the glycine cleavage system genes *gcvP*, *gcvH*, and *gcvT*, the serine biosynthesis gene *serB*, and the cysteine biosynthesis gene *cysE*. All of these mutants displayed normal colonization properties in the infant mouse intestine (Fig. 2).

Disruption of *glyA1* results in glycine auxotrophy but not trimethoprim sensitivity. To phenotypically validate transposon disruption of essential metabolic genes, we examined the methionine mutant panel for growth in M9 minimal medium with glu-

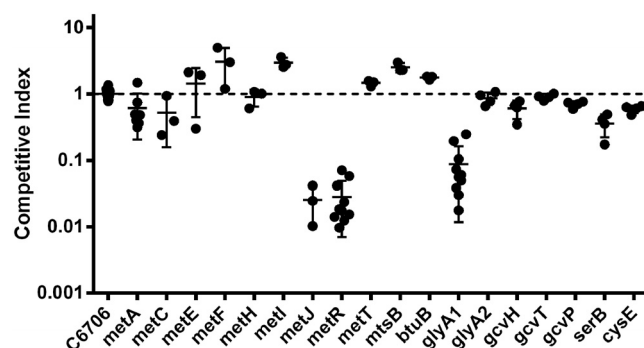


FIG 2 Ability of mutants from the *V. cholerae* metabolism panel to colonize the suckling mouse intestine in competition with wild-type strain C6706. The dotted line indicates a competitive index of 1, and standard deviations (error bars) are presented.

cose as the sole carbon source (see Fig. S1 in the supplemental material). In agreement with their annotated functions, we found that disruption of genes in the homocysteine biosynthesis and folate branches, as well as the cobalamin-independent methionine synthase gene *metE*, resulted in the inability to grow in minimal medium. Furthermore, the cobalamin-dependent methionine synthase gene *metH* and the methionine uptake transporter genes were dispensable, while disruption of the cobalamin uptake transporter gene inhibited growth. The glycine cleavage system genes *gcvH*, *gcvP*, and *gcvT* were not required for growth in minimal medium, while the serine, threonine, cysteine, and homoserine biosynthesis genes *serB*, *thrB*, *cysE*, and *thrA*, respectively, were all required. In agreement with the regulatory roles of MetR and MetJ, disruption of *metR* resulted in lack of growth while the *metJ* mutant exhibited wild-type growth. The *glyA2* mutant grew in minimal medium, consistent with the dispensability of *glyA2* for *in vivo* growth.

The growth kinetics of *glyA1*, *metR*, and *metJ* mutants in M9 minimal medium with and without supplementation with methionine, serine, threonine, aspartate, or glycine (Fig. 3) were analyzed to determine nutritional auxotrophy status of each metabolic mutant. As confirmed previously, MetR and GlyA1 were required for growth in minimal medium, while MetJ was dispensable. The *metJ* mutant exhibited growth inhibition relative to the wild type when glycine was added. The *metR* mutant exhibited methionine auxotrophy that could be reversed with the addition of methionine. In fact, addition of methionine caused increased growth of the *metR* mutant compared to the wild type. The addition of glycine was unable to rescue the *metR* mutant; however, this was likely the result of methionine limitation. The *glyA1* mutant exhibited glycine auxotrophy that could be reversed by the addition of serine, threonine, or glycine but not methionine. Neither the *glyA1* mutant nor the *metR* mutant could be rescued by the addition of aspartate.

GlyA1 not only catalyzes the reversible reaction of serine to glycine but simultaneously regenerates tetrahydrofolate to N^5,N^{10} -methylene tetrahydrofolate by transfer of a methyl group. Disruption of tetrahydrofolate regeneration creates a “methyl trap,” a type of folate deficiency, and consequently growth inhibition. In order to determine if disruption of *glyA1* would result in folate deficiency, we compared trimethoprim sensitivities of the wild type and the *glyA1* mutant. Trimethoprim inhibits dihydrofolate reductase, resulting in blockage of tetrahydrofolic acid synthesis. We hypothesized that if the *in vivo* limitation of tetrahydrofolate was responsible for the colonization defect of the *glyA1* mutant, then this mutant should also exhibit increased sensitivity to, or a reduced MIC of, trimethoprim. The *glyA1* mutant and the wild type exhibited equal sensitivities to trimethoprim, as determined by MIC determination (data not shown) and growth kinetics (see Fig. S2 in the supplemental material).

Chromatin immunoprecipitation of MetR confirms its association with *glyA1*. The regulon of MetR in *V. cholerae* is unknown; thus, a direct regulatory relationship between MetR and *glyA1* is lacking. To determine whether MetR is capable of binding to the promoter of *glyA1*, we performed chromatin immunoprecipitation (ChIP) using epitope-tagged MetR overexpressed in *V. cholerae* from an arabinose-inducible promoter. This method was used in previous studies to map the genomic binding locations of the *V. cholerae* transcriptional regulators Fur and ToxT (11, 28). After conducting ChIP on C6706(pBAD) or

C6706(pMetR-3xV5) grown in LB, we performed quantitative PCR (qPCR) validation to identify enrichment of promoter targets bound by MetR using primers specific to the promoter regions of *metA*, *metE*, *metH*, *metR*, *glyA1*, *glyA2*, *hmpA*, and *tcpA* (Fig. 4). The promoter of toxin-coregulated pilus encoded by *tcpA* served as a negative control. Enrichment of *metE*, *metR*, and *glyA1* promoter regions in C6706(pMetR) relative to C6706(pBAD) were 45-fold, 102-fold, and 45-fold, respectively. Conversely, the promoter regions for *tcpA*, *metA*, *metH*, and *hmpA* were not enriched.

The ChIP results confirmed direct association of MetR with the promoters of *glyA1* and *metE*. To assay MetR transcriptional control at these sites, RNA samples were obtained from C6706(pMetR) incubated in M9 minimal medium or LB medium, with or without arabinose induction of *metR*. The relative abundance of mRNA transcripts from *metA*, *metC*, *metE*, *metH*, *metJ*, *metR*, *glyA1*, *tcpA*, and 16S rRNA transcripts were determined using quantitative reverse transcription-PCR (qRT-PCR). The 16S rRNA served as an internal control to normalize samples to total RNA, and *tcpA* served as a negative control. Our expression analysis compared all samples to the noninduced LB medium sample to yield the relative change in expression (see Table S2 in the supplemental material). We observed 343-fold and 505-fold increases in abundance of *metR* transcript levels under arabinose induction conditions compared to noninduction upon growth in M9 and LB medium, respectively. Furthermore, *metR* induction with arabinose resulted in a 2.0-fold repression of *glyA1* and *metE* in M9 medium and a 3.5-fold upregulation of *metE* in LB medium. All other genes examined showed a <2-fold difference in expression when arabinose induction and noninduction were compared. Analysis of growth in LB relative to M9 medium showed that all genes experienced strong nutrient-dependent repression that ranged from approximately 10-fold to 700-fold regardless of *metR* induction.

DISCUSSION

Transcriptional regulators of the LysR-type family are found in all prokaryotes and regulate virulence in a wide range of bacterial species (29–33). Interestingly, many of the virulence-associated LTTRs found in these bacteria regulate metabolism and virulence factors concomitantly. Of the 38 LTTR mutants screened, only two exhibited an intestinal colonization defect: that with a mutation in the methionine biosynthesis regulator MetR and that with a mutation in a regulator encoded by the locus VC1947, which exhibited a mild *in vivo* defect that was considered significant, although to a much lesser extent than the MetR defect. Upon binding of homocysteine at the N terminus, MetR becomes activated, resulting in transcriptional activation of methionine metabolism genes and negative feedback autorepression of itself (34–36). In pathogenic *E. coli* and *Salmonella enterica* serovar Typhimurium, MetR and the S-adenosylmethionine (SAM)-dependent repressor MetJ are key regulators of a complex metabolite feedback network that maintains proper intracellular methionine levels (36–41); however, regulation of methionine metabolism in *V. cholerae* remains less well understood.

Methionine metabolism is one facet of a highly interconnected and interdependent network of amino acid metabolism (Fig. 5). The biosynthesis of methionine involves two essential pathways: the homocysteine biosynthesis pathway, containing *metA*, *metB*, and *metC*, and the folate pathway, containing *glyA1* and *metF*

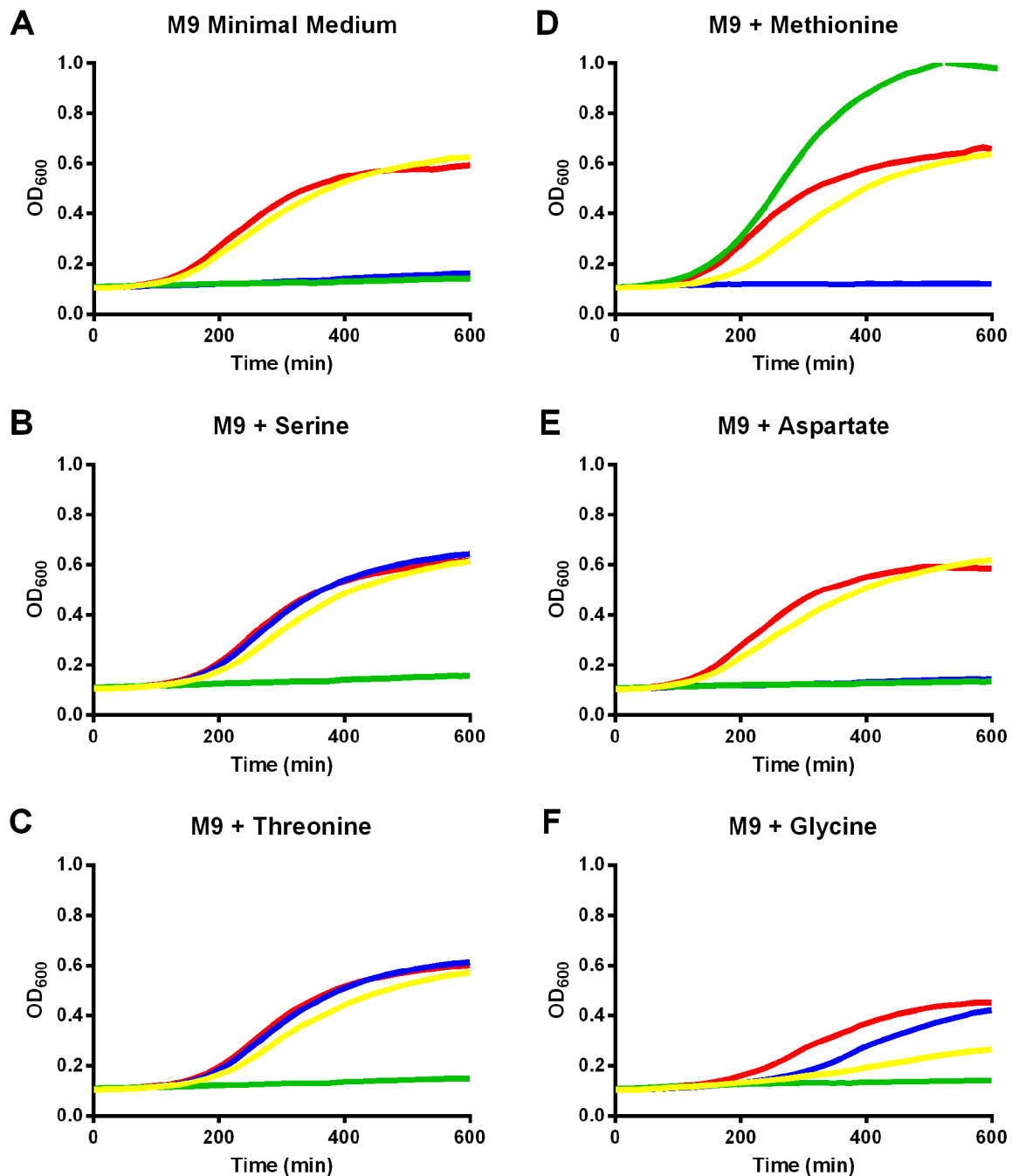


FIG 3 Growth kinetics of wild-type *Vibrio cholerae* (red) and *metR* (green), *metJ* (yellow), and *glyA1* (blue) mutants in M9 minimal medium or M9 supplemented with 0.3 mM concentrations of the indicated amino acids.

(40). The pathways converge at the gene for methionine synthase, *metH* (cobalamin dependent) or *metE* (cobalamin independent), whose product catalyzes the transfer of a methyl group from 5-methyltetrahydrofolate to homocysteine, yielding L-methionine and tetrahydrofolate (40, 42). The work presented here suggests that the SAM-dependent repressor MetJ and serine hydroxymethyltransferase GlyA1 are required for intestinal colonization, while the folate and homocysteine branches of methionine biosynthesis are dispensable *in vivo*. In agreement with this, a previous study

using signature-tagged mutagenesis in *V. cholerae* also identified *glyA1* as being required for colonization of the infant mouse (43). In *E. coli*, MetJ functions as a repressor of methionine biosynthesis genes, including *metR* (41), while MetR is known to activate *glyA1* in a homocysteine-dependent manner (44, 45). Misregulation of *metR* or *glyA1* in the *metJ* repressor mutant may explain its *in vivo* colonization defect. Likewise, misregulation of genes required for virulence, such as *glyA1*, likely explains the colonization defects of the *metR* mutant. As part of the folate branch, GlyA1 catalyzes the

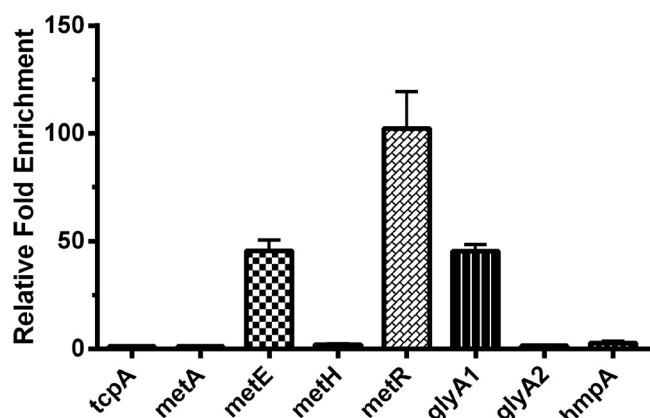


FIG 4 Relative enrichment of promoter targets in ChIP DNA from C6706(pMetR-3xV5) relative to C6706(pBAD) grown in LB medium with arabinose induction.

reversible interconversion of serine to glycine while simultaneously regenerating N^5,N^{10} -methylene tetrahydrofolate (46, 47). The regeneration of methylated tetrahydrofolate is critical not only for the synthesis of methionine but also for a wide array of intracellular methylation events (42, 46, 48). However, the wild-type trimethoprim sensitivity of the *glyA1* mutant indicates that GlyA1 does not play a critical role in the generation of one-carbon (C_1) units, unlike in *E. coli* (49). Taken together, these results

strongly suggest that the production of glycine, rather than methionine or methylated tetrahydrofolate, is required for colonization of the infant mouse intestine by *V. cholerae*.

ChIP analysis of MetR in *V. cholerae* validated its binding capacity to the promoters of genes involved in methionine biosynthesis. As in other bacteria, the loci for *metR* and *metE* are oriented divergently (23), with an ~400-bp intergenic region. Bioinformatic analysis of this intergenic region with the Multiple Expect Maximization for Motif Elicitation (MEME) tool identified a common, but not identical, binding motif that occurs twice (50). The motifs are oriented correctly for divergent transcription of *metR* and *metE* and are separated by ~100 bp. The qPCR primers used in the MetR ChIP analysis were designed to amplify the promoter region (~150 to 200 bp) just upstream of the coding region for each locus; therefore, the fact that *metE* possesses half the enrichment of *metR* indicates differential binding to each promoter. No enrichment for the *meth* promoter was observed; however, the relationship between MetR and its metabolite activator homocysteine is complex and varies greatly with nutrient conditions. In addition to *metE* and *metR*, we confirmed that MetR bound the promoter region of *glyA1* equally well as that of *metE*. Subsequent qRT-PCR analysis supported the ChIP results and validated the regulation of *metE* and *glyA1* by MetR. The regulatory role of MetR was found to be a repressor of *glyA1* and *metE* under M9 medium growth and an activator of *metE* during growth in LB medium. These results are in contrast to the previously reported regulatory control by MetR for *E. coli*; however, regulation in

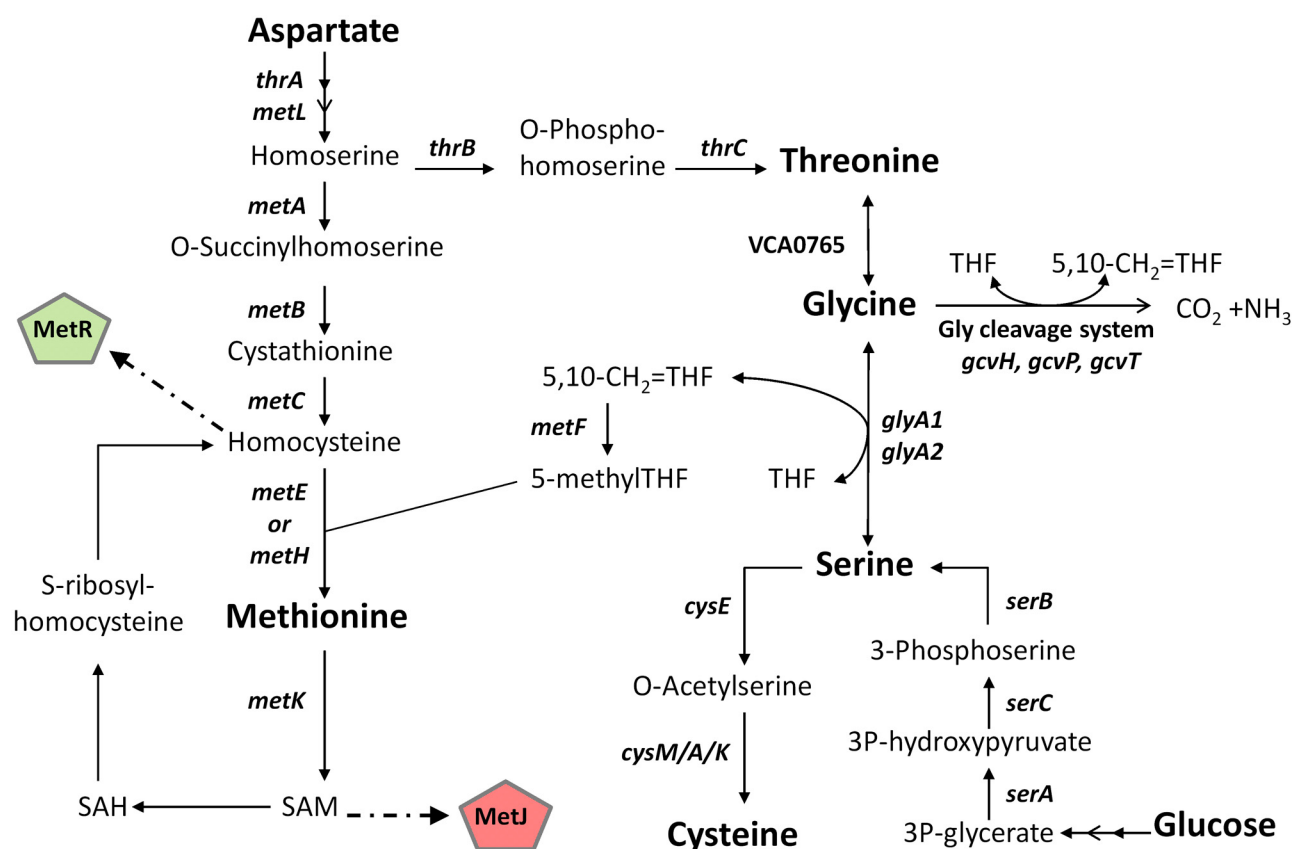


FIG 5 Metabolic pathways of *V. cholerae* showing methionine, aspartate, threonine, glycine, serine, and cysteine biosynthesis. The metabolic transcriptional activator MetR and repressor MetJ are highlighted in green and red, respectively.

V. cholerae may differ. Regulation of amino acid metabolism is carried out with a dynamic network or regulatory circuits with metabolic intermediates serving as coinducers for several transcriptional regulators. In order to maintain amino acid homeostasis, both MetR and MetJ are constantly in flux between induction states but typically reach a state of equilibrium. Another possible explanation for the difference in regulatory control of MetR between our results and those of previous literature is that overexpression of *metR* may result in abnormally high intracellular concentrations that sequester available homocysteine via binding to its coinducer domain. The result would be an artificial reduction in SAM and homocysteine accessibility and subsequently an excess of uninduced, unbound MetJ and/or MetR with unexpected regulatory consequences.

Nutritional opportunities within the host environment require specialized metabolism by the pathogen in order to access and utilize them. Furthermore, these nutritional opportunities may even drive evolutionary pressure to acquire and maintain genetic elements encoding metabolic factors deemed beneficial to survival, replication, or transmission (51). *Vibrio* pathogenicity island 2 (VPI2), encoding a neuraminidase and containing a cluster of genes involved in the scavenging, transport, and utilization of sialic acids, is required for metabolic activity that has been found to influence intestinal colonization (52, 53). While this specialized cluster of metabolic genes was horizontally acquired, other, less specialized metabolic genes from the ancestral genome may be considered to encode virulence factors as well. In this study, we found that glycine biosynthesis by GlyA1 is required by *V. cholerae* for successful colonization of the infant mouse intestine. Intriguingly, the essentiality of GlyA1 and no other related, interdependent, or interconnected metabolic enzymes suggests that glycine is limited in the host intestinal environment, at least relative to its demand by *V. cholerae* for *in vivo* biosynthetic activity or other needs.

Host-microbe metabolism within the mammalian intestine creates a gradient of nutritional niches that as a whole contain a wide diversity of nutrients (54). Interestingly, while most amino acids are rapidly catabolized by the small intestine for fuel, glycine is readily synthesized (55). However, the luminal availability of glycine remains to be determined. A recent *in vivo* transcriptome expression analysis of *V. cholerae* isolated from the rabbit or mouse intestine found no difference in expression of *glyA1* compared to that in the transcriptional profile of LB-grown *V. cholerae* (56). Additionally, in comparisons of expression analyses between conditions (*in vivo* sample versus LB culture; *in vivo* sample versus M9 minimal medium culture), the expression levels of *glyA1* in LB-grown and *in vivo*-isolated *V. cholerae* were similar, while that of M9-cultured *V. cholerae* was approximately 10-fold greater. While *glyA1* was highly expressed under all conditions, the reduced expression of *glyA1* *in vivo* relative to that in minimal medium suggests adequate glycine availability in the host intestinal environment. This raises the possibility that glycine biosynthesis may play additional roles in virulence besides fulfillment of nutritional requirements. Bacteria are known to export amino acids to the extracellular environment via specific transporters (57, 58), and excretion of glycine by *E. coli* has been shown to elicit a quorum-based social behavior (59). The chemoreceptors Tar and Tsr sense serine and glycine or aspartate, respectively, and the bacteria respond collectively by congregation or dispersion depending upon temperature (60). Interestingly, several amino acids,

including glycine, have been found to bind to chemoreceptors in *V. cholerae* and serve as strong chemotactic attractants (61). One of these chemoreceptors, Mlp24 (McpX), is required for expression of *ctxAB* in the infant mouse model; however, the underlying mechanism of genetic control is unknown (62). It is interesting to speculate that *V. cholerae* may possess a glycine excretion and response system similar to *E. coli* that coordinates chemotactic responses required for virulence. The dependency of glycine biosynthesis for host colonization by *V. cholerae* El Tor highlights the importance of understanding the *in vivo* metabolism of pathogens.

MATERIALS AND METHODS

Ethics statement. The animal experiments were performed according to protocols approved by Harvard Medical School Office for Research Protection Standing Committee on Animals. The Harvard Medical School animal management program is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International (AAALAC) and meets National Institutes of Health standards as set forth in the *Guide for the Care and Use of Laboratory Animals* (63). The institution also accepts as mandatory the PHS Policy on Humane Care and Use of Laboratory Animals by Awardee Institutions and NIH Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training. There is on file with the Office of Laboratory Animal Welfare (OLAW) an approved Assurance of Compliance (A3431-01).

Bacterial strains. Strains and plasmids are listed in Table S3A in the supplemental material. *V. cholerae* El Tor biotype strain C6706 and a spontaneous *lacZ* derivative of C6706 were used as parental (wild-type [WT]) strains. *E. coli* DH5 α λ pir was used for cloning. Antibiotics used were streptomycin (Sm; 100 μ g/ml), kanamycin (Kan; 50 μ g/ml), trimethoprim (Tnp; ~39 ng/ml to 5 μ g/ml), and chloramphenicol (Cm; 2.5 μ g/ml for C6706 and 10 μ g/ml for *E. coli* DH5 α λ pir). LB was used for normal growth conditions [10 g/liter of tryptone (Bacto), 5 g/liter of yeast extract (Bacto), and 5 g/liter of NaCl] and was supplemented with 16 g/liter of agar (Bacto) for growth on plates. Filter-sterilized M9 minimal medium was used for auxotrophy determination assays [11.28 g/liter M9 minimal salts (prepared as instructed by the manufacturer; Sigma), 20 mM glucose, 2 mM MgSO₄, and 0.1 mM CaCl₂]. Arabinose was used at a final concentration of 0.02% for ChIP and *metR* expression experiments. X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) was used at 40 mg/ml.

DNA manipulations and cloning. To construct pMetR or pMetR-3xV5, the LysR-type transcriptional regulator *metR* was PCR amplified (NEB Phusion HF Master Mix) from *V. cholerae* genomic DNA and cloned into plasmid pBAD18 or pBAD18 carrying a C-terminal 3 \times V5 epitope tag, respectively, after digestion with KpnI and SalI (NEB). All cloned products were sequence verified (Dana Farber). Primers are listed in Table S3B in the supplemental material. The vectors pMetR and pMetR-3xV5 were then electroporated using standard techniques into *V. cholerae* C6706 *metR::Tn* to generate C6706(pMetR) and C6706(pMetR-3xV5), respectively.

Suckling mouse colonization competition assays. A modified version of the protocol described by Sigel et al. (22) was performed for infection and recovery of C6706-derived strains. C6706, C6706 *lacZ*, and transposon mutant strains were grown on LB agar plates with Sm overnight at 37°C. Wild-type and mutant strains (10 μ l each) were mixed together in 1 ml LB. Fifty microliters of this competition mixture (~50,000 bacteria) was inoculated into a 5-day-old CD1 mouse pup (Charles River Company). Serial dilutions of the competition mixture were plated on LB containing Sm (LB+Sm¹⁰⁰) and enumerated to determine the input ratio of wild-type and mutant strains. After incubation at 30°C for 12 h, the mouse pups were sacrificed, and small intestines were removed and homogenized in 10 ml of LB. Serial dilutions were plated on LB+Sm¹⁰⁰ and enumerated to determine the output ratio of wild-type and mutant strains. The competitive index for each mutant is defined as the input ratio

of mutant to wild-type strains divided by the output ratio of mutant to wild-type strains. On average, four mice were assayed for each mutant strain. The results of *in vivo* experiments for the transposon strains were the cumulative results of experiments performed on different days. The average competitive index was reported for ease of communication.

Growth in minimal medium with or without amino acid supplementation. Overnight cultures were diluted (1:1,000) in LB and grown to log phase at 30°C with aeration. One milliliter of culture was pelleted by centrifugation, washed 3× in M9 minimal medium, and then diluted to an optical density at 600 nm (OD₆₀₀) of 0.1 in M9 minimal medium with or without supplementation with 0.3 mM methionine, aspartate, threonine, glycine, or serine. Auxotrophy was determined by the absence or presence of growth. Growth kinetics of strains in LB or M9 minimal medium with or without amino acids was determined by OD₆₀₀ measurement using a 96-well-plate-format spectrophotometer (Spectramax Plus 384; Molecular Devices). Environmental parameters were set to 30°C with aeration, and readings were taken every 15 min for 10 h. Studies were conducted in triplicate.

Trimethoprim sensitivity assay. Overnight cultures were diluted (1:1,000) in LB and grown to log phase at 30°C with aeration. Cultures were diluted to an OD₆₀₀ of 0.1 in fresh LB containing twofold serially diluted trimethoprim (1 µg/µl). Sensitivity to trimethoprim, as determined by growth kinetics of mutant strains relative to WT, was determined by OD₆₀₀ measurement using a 96-well-plate-format spectrophotometer (Spectramax Plus 384; Molecular Devices). Environmental parameters were set to 30°C with aeration, and readings were taken every 15 min for 10 h. Studies were conducted in triplicate. Trimethoprim MIC determination was conducted by washing cells and diluting them (1:100) in 5 ml LB with twofold serial dilutions of trimethoprim. Cultures were grown at 30°C with aeration and monitored for the presence or absence of growth, as indicated by turbidity, after 36 h.

Chromatin immunoprecipitation (ChIP). ChIP was carried out as previously described (11, 28); however, changes were made to optimize the protocol. Fifty milliliters of exponentially growing culture [C6706(pMetR-3xV5)] in LB was induced with 0.02% arabinose for 30 min at 37°C with aeration. Formaldehyde (37%, Sigma) was added to a final concentration of 1% and incubated at RT for 20 min with occasional swirling. Cross-linking was quenched by adding glycine to 0.5 M. Cell pellets were washed in 1× Tris-buffered saline (TBS), resuspended in lysis buffer [10 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% deoxycholate (DOC), 0.5% *N*-lauroylsarcosine] plus bacterial protease inhibitor cocktail (Sigma P8465) and 50,000 U Ready-Lyse lysozyme (Epicentre), and then incubated at 30°C for 30 min. The cells were subjected to needle sonication for 60 s and unlysed debris was removed by centrifugation. To shear DNA, the lysate was sonicated for 10 min with a 10-s on/10-s off cycle (Mixonix). A sample was taken as a sequencing input control. Following clarification by centrifugation, 1/10 volume of 10% Triton X-100 in lysis buffer was added to each sample followed by 50 µl of Dynal-Protein G beads coated with anti-V5 monoclonal antibody (Sigma), and samples were incubated overnight at 4°C with rotation. The beads were washed five times with radioimmunoprecipitation assay (RIPA) buffer [50 mM HEPES (pH 7.5), 500 mM LiCl, 1 mM EDTA, 1% Nonidet P-40, 0.7% deoxycholate (DOC)] and once with Tris-EDTA (pH 8.0) plus 50 mM NaCl and then resuspended in 100 µl elution buffer [50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 1% sodium dodecyl sulfate (SDS)]. Samples were incubated at 65°C for 30 min, and then the beads were removed by magnet. Supernatants were incubated at 65°C overnight to reverse cross-links. Each sample's volume was increased by the addition of 100 µl Tris-EDTA (pH 8.0), and then samples were incubated with 8 µl of 10 mg/ml RNase A for 2 h at 37°C. Samples were then treated with 4 µl of 20 mg/ml proteinase K at 55°C for 2 h, purified with a MinElute reaction cleanup kit (Qiagen), eluted into 50 µl, and quantified with a Pico green kit (Invitrogen). Experiments were performed in triplicate.

Quantitative PCR and RT-PCR. For ChIP-Seq peak validation, relative-abundance quantitative PCR (qPCR) was performed with Kapa

Biosystems Fast SYBR green Master Mix using 16S rRNA genes and *tcpA* targets as controls. Relative target levels were calculated using the $\Delta\Delta C_T$ method, with normalization of ChIP targets to the 16S rRNA gene signal (64). For gene expression analysis, total RNA was extracted using a RiboPure kit (Invitrogen), contaminating DNA removed using a Turbo DNA-free kit (Ambion), quantification was conducted on a NanoDrop spectrophotometer (Thermo Scientific), and abundance was normalized among samples. Relative-expression qRT-PCR was performed with a Power SYBR green RNA-to- C_T one-step kit (Applied Biosystems). Relative expression levels were calculated using the $\Delta\Delta C_T$ method with normalization of gene targets to the 16S rRNA signal (64). For qRT-PCR experiments, cells of C6706(pMetR) were grown to log phase in 5 ml of LB medium at 37°C, washed three times in M9 minimal medium, resuspended in 5 ml of M9 medium, incubated at 37°C for 1 h with aeration, and then induced with arabinose for 30 min at 37°C, and RNA was extracted. Primers used are listed in Table S3B in the supplemental material.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00236-12/-DCSupplemental>.

Figure S1, TIF file, 10.7 MB.

Figure S2, TIF file, 2.6 MB.

Table S1, XLSX file, 0.1 MB.

Table S2, XLSX file, 0.1 MB.

Table S3, XLSX file, 0.1 MB.

ACKNOWLEDGMENTS

We thank Ewen Cameron for use of the *V. cholerae* sequence defined transposon library (20).

B.W.D. was supported by the Jane Coffin Childs Memorial Fund for Medical Research and by the National Research Council of Canada H. L. Holmes Award. This work was supported by the National Institutes of Health Grant AI-018045 to J.J.M.

REFERENCES

1. Faruque SM, Albert MJ, Mekalanos JJ. 1998. Epidemiology, genetics, and ecology of toxigenic *Vibrio cholerae*. Microbiol. Mol. Biol. Rev. 62: 1301–1314.
2. Morris JG, Black RE. 1985. Cholera and other vibrioses in the United States. N. Engl. J. Med. 312:343–350.
3. World Health Organization (WHO). July 2012. Cholera factsheet. WHO, Geneva, Switzerland. <http://www.who.int/mediacentre/factsheets/fs107/en/index.html>.
4. Kaper JB, Morris JG, Levine MM. 1995. Cholera. Clin. Microbiol. Rev. 8:48–86.
5. Taylor RK, Miller VL, Furlong DB, Mekalanos JJ. 1987. Use of *phoA* gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. Proc. Natl. Acad. Sci. U S A 84:2833–2837.
6. Faruque SM, Mekalanos JJ. 2003. Pathogenicity islands and phages in *Vibrio cholerae* evolution. Trends Microbiol. 11:505–510.
7. Waldor MK, Mekalanos JJ. 1996. Lysogenic conversion by a filamentous phage encoding cholera toxin. Science 272:1910–1914.
8. Mekalanos JJ, et al. 1983. Cholera toxin genes: nucleotide sequence, deletion analysis and vaccine development. Nature 306:551–557.
9. Butler SM, et al. 2006. Cholera stool bacteria repress chemotaxis to increase infectivity. Mol. Microbiol. 60:417–426.
10. Davies BW, et al. 2011. DNA damage and reactive nitrogen species are barriers to *Vibrio cholerae* colonization of the infant mouse intestine. PLoS Pathog. 7:e1001295.
11. Davies BW, Bogard RW, Young TS, Mekalanos JJ. 2012. Coordinated regulation of accessory genetic elements produces cyclic di-nucleotides for *V. cholerae* virulence. Cell 149:358–370.
12. Faruque SM, et al. 2006. Transmissibility of cholera: *in vivo*-formed biofilms and their relationship to infectivity and persistence in the environment. Proc. Natl. Acad. Sci. U S A 103:6350–6355.
13. Beier D, Gross R. 2006. Regulation of bacterial virulence by two-component systems. Curr. Opin. Microbiol. 9:143–152.
14. Champion GA, Neely MN, Brennan MA, DiRita VJ. 2003. A branch in

- the ToxR regulatory cascade of *Vibrio cholerae* revealed by characterization of toxT mutant strains. *Mol. Microbiol.* 23:323–331.
15. Herrington DA, et al. 1988. Toxin, toxin-coregulated pili, and the toxR regulon are essential for *Vibrio cholerae* pathogenesis in humans. *J. Exp. Med.* 168:1487–1492.
 16. Henikoff S, Haugn GW, Calvo JM, Wallace JC. 1988. A large family of bacterial activator proteins. *Proc. Natl. Acad. Sci. U S A* 85:6602–6606.
 17. Knapp GS, Hu JC. 2010. Specificity of the *E. coli* LysR-type transcriptional regulators. *PLoS One* 5:e15189.
 18. Maddocks SE, Oyston PC. 2008. Structure and function of the LysR-type transcriptional regulator (LTTR) family proteins. *Microbiology* 154:3609–3623.
 19. Schell MA. 1993. Molecular biology of the LysR family of transcriptional regulators. *Annu. Rev. Microbiol.* 47:597–626.
 20. Cameron DE, Urbach JM, Mekalanos JJ. 2008. A defined transposon mutant library and its use in identifying motility genes in *Vibrio cholerae*. *Proc. Natl. Acad. Sci. U S A* 105:8736–8741.
 21. Klose KE. 2000. The suckling mouse model of cholera. *Trends Microbiol.* 8:189–191.
 22. Sigel SP, Lanier S, Baselski VS, Parker CD. 1980. *In vivo* evaluation of pathogenicity of clinical and environmental isolates of *Vibrio cholerae*. *Infect. Immun.* 28:681–687.
 23. Augustus AM, Spicer LD. 2011. The MetJ regulon in *Gammaproteobacteria* determined by comparative genomics methods. *BMC Genomics* 12:558.
 24. Heidelberg JF, et al. 2000. DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. *Nature* 406:477–483.
 25. Kanehisa M, Goto S, Sato Y, Furumichi M, Tanabe M. 2012. KEGG for integration and interpretation of large-scale molecular data sets. *Nucleic Acids Res.* 40:D109–D114.
 26. Kanehisa M, Goto S. 2000. KEGG: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Res.* 28:27–30.
 27. Shi J, Romero PR, Schoolnik GK, Spormann AM, Karp PD. 2006. Evidence supporting predicted metabolic pathways for *Vibrio cholerae*: gene expression data and clinical tests. *Nucleic Acids Res.* 34:2438–2444.
 28. Davies BW, Bogard RW, Mekalanos JJ. 2011. Mapping the regulon of *Vibrio cholerae* ferric uptake regulator expands its known network of gene regulation. *Proc. Natl. Acad. Sci. U S A* 108:12467–12472.
 29. Allen JP, Neely MN. 2011. The *Streptococcus iniae* transcriptional regulator CpsY is required for protection from neutrophil-mediated killing and proper growth in vitro. *Infect. Immun.* 79:4638–4648.
 30. Bryan JD, Liles R, Cvek U, Trutschl M, Shelper D. 2008. Global transcriptional profiling reveals *Streptococcus agalactiae* genes controlled by the MtaR transcription factor. *BMC Genomics* 9:607.
 31. Easom CA, Clarke DJ. 2012. HdfR is a regulator in *Photobacterium luminescens* that modulates metabolism and symbiosis with the nematode *Heterorhabditis*. *Environ. Microbiol.* 14:953–966.
 32. Wei Q, et al. 2012. Global regulation of gene expression by OxyR in an important human opportunistic pathogen. *Nucleic Acids Res.* 40:4320–4333.
 33. Yeung AT, et al. 2009. Swarming of *Pseudomonas aeruginosa* is controlled by a broad spectrum of transcriptional regulators, including MetR. *J. Bacteriol.* 191:5592–5602.
 34. LaMonte BL, Hughes JA. 2006. *In vivo* hydrolysis of S-adenosylmethionine induces the met regulon of *Escherichia coli*. *Microbiology* 152:1451–1459.
 35. Urbanowski ML, Stauffer LT, Plamann LS, Stauffer GV. 1987. A new methionine locus, metR, that encodes a *trans*-acting protein required for activation of metE and metH in *Escherichia coli* and *Salmonella typhimurium*. *J. Bacteriol.* 169:1391–1397.
 36. Wu WF, Urbanowski ML, Stauffer GV. 1992. Role of the MetR regulatory system in vitamin B12-mediated repression of the *Salmonella typhimurium* metE gene. *J. Bacteriol.* 174:4833–4837.
 37. Cowan JM, Urbanowski ML, Talmi M, Stauffer GV. 1993. Regulation of the *Salmonella typhimurium* metF gene by the MetR protein. *J. Bacteriol.* 175:5862–5866.
 38. Shoeman R, et al. 1985. Regulation of methionine synthesis in *Escherichia coli*: effect of metJ gene product and S-adenosylmethionine on the expression of the metF gene. *Proc. Natl. Acad. Sci. U S A* 82:3601–3605.
 39. Su CH, Greene RC. 1971. Regulation of methionine biosynthesis in *Escherichia coli*: mapping of the metJ locus and properties of a metJ plus-metJ minus diploid. *Proc. Natl. Acad. Sci. U S A* 68:367–371.
 40. Weissbach H, Brot N. 1991. Regulation of methionine synthesis in *Escherichia coli*. *Mol. Microbiol.* 5:1593–1597.
 41. Wu WF, Urbanowski ML, Stauffer GV. 1993. MetJ-mediated regulation of the *Salmonella typhimurium* metE and metR genes occurs through a common operator region. *FEMS Microbiol. Lett.* 108:145–150.
 42. Matthews RG, Drummond JT, Webb HK. 1998. Cobalamin-dependent methionine synthase and serine hydroxymethyltransferase: targets for chemotherapeutic intervention? *Adv. Enzyme Regul.* 38:377–392.
 43. Merrell DS, Hava DL, Camilli A. 2002. Identification of novel factors involved in colonization and acid tolerance of *Vibrio cholerae*. *Mol. Microbiol.* 43:1471–1491.
 44. Lorenz E, Stauffer GV. 1995. Characterization of the MetR binding sites for the glyA gene of *Escherichia coli*. *J. Bacteriol.* 177:4113–4120.
 45. Plamann MD, Stauffer GV. 1989. Regulation of the *Escherichia coli* glyA gene by the metR gene product and homocysteine. *J. Bacteriol.* 171:4958–4962.
 46. Chen MS, Schirch LV. 1973. Serine transhydroxymethylase: studies on the role of tetrahydrofolate. *J. Biol. Chem.* 248:7979–7984.
 47. Scarsdale JN, Radaev S, Kazanina G, Schirch V, Wright HT. 2000. Crystal structure at 2.4 Å resolution of *E. coli* serine hydroxymethyltransferase in complex with glycine substrate and 5-formyl tetrahydrofolate. *J. Mol. Biol.* 296:155–168.
 48. Appaji Rao N, Talwar R, Savithri HS. 2000. Molecular organization, catalytic mechanism and function of serine hydroxymethyltransferase—a potential target for cancer chemotherapy. *Int. J. Biochem. Cell Biol.* 32:405–416.
 49. Dev IK, Harvey RJ. 1982. Sources of one-carbon units in the folate pathway of *Escherichia coli*. *J. Biol. Chem.* 257:1980–1986.
 50. Bailey TL, Williams N, Misleh C, Li WW. 2006. MEME: discovering and analyzing DNA and protein sequence motifs. *Nucleic Acids Res.* 34:W369–W373.
 51. Rohmer L, Hocquet D, Miller SI. 2011. Are pathogenic bacteria just looking for food? Metabolism and microbial pathogenesis. *Trends Microbiol.* 19:341–348.
 52. Chowdhury N, et al. 2012. The VC1777-VC1779 proteins are members of a sialic acid-specific subfamily of TRAP transporters (SiaPQM) and constitute the sole route of sialic acid uptake in the human pathogen *Vibrio cholerae*. *Microbiology* 158:2158–2167.
 53. Jermyn WS, Boyd EF. 2002. Characterization of a novel vibrio pathogenicity island (VPI-2) encoding neuraminidase (nanH) among toxigenic *Vibrio cholerae* isolates. *Microbiology* 148:3681–3693.
 54. Hooper LV, Midtvedt T, Gordon JI. 2002. How host-microbial interactions shape the nutrient environment of the mammalian intestine. *Annu. Rev. Nutr.* 22:283–307.
 55. Wu G. 1998. Intestinal mucosal amino acid catabolism. *J. Nutr.* 128:1249–1252.
 56. Mandlik A, et al. 2011. RNA-Seq-based monitoring of infection-linked changes in *Vibrio cholerae* gene expression. *Cell Host Microbe* 10:165–174.
 57. Eggeling L, Sahm H. 2003. New ubiquitous translocators: amino acid export by *Corynebacterium glutamicum* and *Escherichia coli*. *Arch. Microbiol.* 180:155–160.
 58. Hori H, et al. 2011. Inducible l-alanine exporter encoded by the novel gene *ygaW* (*alaE*) in *Escherichia coli*. *Appl. Environ. Microbiol.* 77:4027–4034.
 59. Park S, et al. 2003. Influence of topology on bacterial social interaction. *Proc. Natl. Acad. Sci. U S A* 100:13910–13915.
 60. Salman H, Libhaber A. 2007. A concentration-dependent switch in the bacterial response to temperature. *Nat. Cell Biol.* 9:1098–1100.
 61. Nishiyama S, et al. 2012. Mlp24 (McpX) of *Vibrio cholerae* implicated in pathogenicity functions as a chemoreceptor for multiple amino acids. *Infect. Immun.* 80:3170–3178.
 62. Lee SH, Butler SM, Camilli A. 2001. Selection for in vivo regulators of bacterial virulence. *Proc. Natl. Acad. Sci. U S A* 98:6889–6894.
 63. National Research Council. 1996. Guide for the care and use of laboratory animals. National Academies Press, Washington, DC.
 64. Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{−ΔΔCT} method. *Methods* 25:402–408.